

MECHANISMS UNDERLYING THE SPERM QUALITY ADVANTAGE IN *DROSOPHILA MELANOGASTER*

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Abstract.—Contrary to early predictions of sperm competition theory, postcopulatory sexual selection favoring increased investment per sperm (e.g., sperm size, sperm quality) has been demonstrated in numerous organisms. We empirically demonstrate for *Drosophila melanogaster* that both sperm quality and sperm quantity independently contribute to competitive male fertilization success. In addition to these independent effects, there was a significant interaction between sperm quality and quantity that suggests an internal positive reinforcement on selection for sperm quality, with selection predicted to intensify as investment per sperm increases and the number of sperm competing declines. The mechanism underlying the sperm quality advantage is elucidated through examination of the relationship between female sperm-storage organ morphology and the differential organization of different length sperm within the organ. Our results exemplify that primary sex cells can bear secondary sexual traits.

Key words.—Cryptic female choice, postcopulatory sexual selection, sperm competition, sperm quality, sperm size.

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Disruptive selection acting on an isogamous population serves as a popular theory for the origin of anisogamy (Parker et al. 1972; Bulmer and Parker 2002). Sperm competition theory applies the same selective conditions (i.e., the more numerically abundant gamete type competing to fuse with the rarer gamete type) to explain the evolutionary maintenance of anisogamy (Parker 1982). Specifically, most theoretical treatments model sperm competition as a raffle, with the probability of a given male siring an offspring depending on the relative representation of his sperm in the “fertilization set” (Parker 1970a, 1982, 1984, 1990a,b; Parker et al. 1972, 1996, 1997; Williams et al. 2005). Under these conditions, males will be selected to invest minimally in each sperm (i.e., tiny sperm) and thus maximize the number of sperm produced (e.g., Parker 1970a, 1982, 1984, 1990a,b; Parker et al. 1972).

All other things being equal, greater sperm numbers should nearly always enhance male fertilization success (with the possible exception of species in which males can efficiently remove, incapacitate, or displace previously stored sperm; e.g., Waage 1979). This prediction has received robust empirical support. First, experiments with numerous taxa have demonstrated that males copulating longer or transferring larger ejaculates or greater numbers of sperm achieve paternity (Birkhead and Møller 1998; Simmons 2001). Second, this sperm quantity advantage certainly underlies the taxonomically widespread relationship between relative testis mass and the intensity of sperm competition demonstrated through comparative analyses (e.g., Harcourt et al. 1981; Pitcher et al. 2005; Ramm et al. 2005). Third, males from populations for which sexual selection has been experimentally eliminated evolve relatively smaller testes (Hosken and Ward 2001; Pitnick et al. 2001).

Nevertheless, things are not always equal (Snook 2005). For example, among insects, sperm quality, as measured by sperm viability, positively covaries with the intensity of sperm competition (Hunter and Birkhead 2002). Also, experiments controlling the number of sperm inseminated into females have found repeatable and/or heritable differences

among males in ejaculate performance or the outcome of sperm competition (Martin et al. 1974; Dziuk 1996; Birkhead et al. 1999; Froman et al. 2002). Sperm cells are the most diverse cell type known, exhibiting rapid and dramatic evolutionary divergence in form (Sivinski 1984; Baccetti 1986; Jamieson 1991; Pitnick et al. 1995a, 2003; Jamieson et al. 1999; Morrow 2004), as expected of traits subject to intense sexual selection (Eberhard 1985; Andersson 1994). Although the evolution of sperm quality traits by postcopulatory sexual selection has been suggested numerous times (Roldan 1991; Roldan et al. 1992; Birkhead and Møller 1993; Birkhead et al. 1993; Gomendio and Roldan 1993; Eberhard 1996; Birkhead and Pizzari 2002; Pizzari and Birkhead 2002; Snook 2005), the adaptive significance of nearly all variation in sperm form or quality that has been the subject of widespread evolutionary investigation is size.

Theoretical treatments of sperm size evolution have approached the problem from a parental investment theory perspective (exceptions discussed below), with the principal adaptive benefit of larger sperm being enhanced zygote viability (Parker 1982, 1984; Parker et al. 1972; Trivers 1972; Bulmer and Parker 2002). Such models indicate that, with a starting condition of extreme anisogamy, an increase in sperm size will only be favored when there is no sperm competition. Parker (1982, p. 287) summarizes the conclusion as follows: “Essentially, the reason it does not pay to increase sperm provisioning is that a unit increase in investment in each sperm causes significant cost, but insignificant benefit. For example, doubling the sperm size halves the sperm number, which causes significant losses when there is sperm competition. But doubling the sperm size would effect a virtually insignificant increase in the viability of the zygote.”

We contend that parental investment theory provides a limited perspective for considering sperm size evolution. It is true that the entire sperm cell enters the egg in the majority of species (Ankel-Simons and Cummins 1996; Karr and Pitnick 1996; Snook and Karr 1998) and that postfertilization interaction between the sperm and the egg can be protracted

and complex (Karr 1991; Pitnick and Karr 1998). It is also now recognized that the sperm contributes more essential product to the zygote than simply the haploid complement of paternal DNA (e.g., the centrosome, a variety of proteins and RNA; Schatten 1994; Karr 1996; Churchill et al. 2003; Krawetz 2005; Loppin et al. 2005; Rauh et al. 2005; Rasoulzadegan et al. 2006). Unfortunately, very little is known about fertilization in animals other than chordates and echinoderms (Sander 1985), and even less is known about the fate of sperm-derived products following fertilization (Karr 1996; Pitnick and Karr 1998; Krawetz 2005). Nevertheless, the only relevant study to date strongly suggests that post-fertilization function is not the driving force behind evolutionary diversification of sperm size (Karr and Pitnick 1996). Thus, although postzygotic traits were explicitly considered by Trivers (1972) as parental investment, we contend that sperm “quality” attributes arising from postcopulatory sexual selection represent energies expended in intrasexual competition and intersexual choice, and hence are specifically excluded from parental investment by Trivers (1972).

On the other hand, it was recently suggested that the long sperm tails of *Drosophila* are the cellular, postcopulatory equivalent of peacock tails (Miller and Pitnick 2002). A compelling body of evidence supports this contention (Keller and Reeve 1995; Snook 2005). First, intraspecific variation in sperm size positively correlates with fertilization success in the bulb mite *Rhizoglyphus robini* (Radwan 1996), the nematode *Caenorhabditis elegans* (LaMunyon and Ward 1998), and the freshwater snail *Viviparus ater* (Oppliger et al. 2003). Second, selection lines of *C. elegans* evolved larger sperm in response to experimentally increased levels of sperm competition (LaMunyon and Ward 2002), and males from lines of the fruit fly, *D. melanogaster*, experimentally selected to have longer sperm demonstrated enhanced competitive fertilization success (Miller and Pitnick 2002; but see Gage and Morrow 2003, discussed in detail below). Third, comparative studies of a diverse array of taxa have found a significant positive relationship between sperm length and the risk or intensity of sperm competition (mammals: Gomendio and Roldan 1991; primates: Dixson 1993; birds: Briskie and Montgomerie 1992; Briskie et al. 1997; Johnson and Briskie 1999; butterflies: Gage 1994; nematodes: LaMunyon and Ward 1999; moths: Morrow and Gage 2000; cichlid fish: Balshine et al. 2001; frogs: Byrne et al. 2003; rodents: Breed 2004; but for exceptions see Stockley et al. 1997 on fish and Harcourt 1991; Hosken 1997; Anderson and Dixson 2002; Gage and Freckleton 2003 on mammals, discussed in detail below). Fourth, comparative studies on diverse taxa have found significant correlated evolution between sperm length and dimensions of some critical region of the female reproductive tract (featherwing beetles: Dybas and Dybas 1981; birds: Briskie and Montgomerie 1993; fruit flies: Pitnick et al. 1999, 2003; stalk-eyed flies: Presgraves et al. 1999; moths: Morrow and Gage 2000; dung flies: Minder et al. 2005; but for megachiropteran bats see Hosken 1998). The interpretation that this correlation results from sperm size evolving in response to changing female reproductive tract design is supported by an experimental evolution study showing that evolving female sperm-storage organ morphology can drive

the evolution of sperm length (Miller and Pitnick 2002, 2003).

Because antagonistic pleiotropy between sperm size and the number of sperm produced is expected (e.g., Parker 1982) and has been empirically demonstrated (Pitnick 1996; Oppliger et al. 1998), it would be informative to know how these two traits interact to determine male competitive fertilization success (Gage and Morrow 2003). The quandary over how postcopulatory sexual selection acts on sperm quality and quantity is further complicated by the centrality of Bateman’s (1948) contribution to sexual selection theory. Bateman’s quantitative description of sex differences in *D. melanogaster* gave rise to the modern era of sexual selection theory (Trivers 1972; Emlen and Oring 1977; Clutton-Brock and Parker 1992; Shuster and Wade 2003) by showing that the slope of the line relating reproductive success to mating success (the sexual selection gradient) is nearly flat for females, whereas the slope of this line is much steeper for males. The magnitude of the sex difference in the strength of selection is believed to depend upon the relationship between male and female sexual selection gradients (Jones et al. 2000, 2002). Anisogamy generates the conditions for sexual selection, as numerically abundant male gametes compete to fertilize rare female gametes (Kokko and Jennions 2003). For the majority of species, (those lacking postfertilization parental investment; e.g., most *Drosophila*; Pitnick et al. 1997), the intensity of sexual selection distills down to the sex difference in the number of gametes produced. Because sperm size and number trade off (Pitnick 1996; Oppliger et al. 1998), the evolution of giant sperm by sexual selection is an apparent paradox: as sperm size increases, sperm become less abundant, ova become relatively less rare, and hence competition between sperm (or males) for fertilization success is predicted to weaken. As a consequence, theory predicts an inverse relationship between sperm size and the intensity of sexual selection, whereas empirical investigations (cited above) suggest that relatively large sperm are the result of more intense sexual selection (the “big sperm paradox”; Bjork and Pitnick 2006).

What is needed to clarify our understanding of sexual selection for sperm quality, and to discern whether certain sperm characters are secondary sexual traits, is (1) an understanding of the relationship between sperm quality (e.g., size) and the intensity of sexual selection, (2) knowledge of how sperm quality and quantity contribute to the pattern of sperm precedence, (3) elucidation of the mechanisms by which sperm and the female reproductive tract interact to generate selection on sperm quality, and (4) identification of the selective benefits accrued by females from choosing among sperm. By repeating Bateman’s (1948) experiments with species of *Drosophila*, as well as with experimental evolution lines of *D. melanogaster* that differ in sperm length (Miller and Pitnick 2002), we have recently made progress toward the first goal by demonstrating that the opportunity for sexual selection does not decrease with increasing sperm length (Bjork and Pitnick 2006). Herein, working with the same lines of *D. melanogaster*, we report the results of experiments fulfilling the second and third goals (but not the fourth goal). Specifically, using a fully factorial design, we investigate the effect of varying sperm length and sperm num-

ber on second male sperm precedence. Next, we provide a detailed examination of the distribution of sperm within the primary sperm-storage organ, revealing a pattern of organization that corresponds to the architecture of the female organ. Finally, we quantify the distribution of competing short and long sperm within females to reveal some of the mechanisms by which males with relatively long sperm achieve a fertilization advantage.

MATERIALS AND METHODS

Experimental Populations and Culturing

All experiments were conducted on populations of *D. melanogaster* artificially selected bidirectionally for either sperm length or seminal receptacle (SR) length. Details of the selection protocols and of the source populations are provided in Miller and Pitnick (2002, 2003). Males were from short-sperm or long-sperm populations, 36–48 generations following the inception of selection on sperm length. Females were from short-SR or long-SR populations (replicate B), 58–60 generations following the inception of selection on SR length. Note, however, that these populations have not been subject to selection for sperm or SR length since generations 17 and 38, respectively. Nevertheless, as demonstrated by data presented herein, no appreciative regression of the traits has occurred. It is also important to note that there exists only a single replicate of the experimental evolution sperm length-lines and that females from only a single replicate of the experimental evolution SR-length lines were used in the present study. Consequently, for some evolutionary questions, the present paper reports a sample size of $n = 1$.

Additionally, for the sperm competition experiment, LH_M - BW strain males were used. This strain was derived from a large outbred population (LH_M) that had adapted to the laboratory for over 200 generations, and carries a brown-eyed (BW) dominant marker that had been introgressed through 12–13 backcross generations into the LH_M background (for details on the origin and maintenance of these lines see Chipindale et al. 2001). These lines were obtained from A. Chipindale (Dept. of Biology, Queen's University, Canada) and maintained in our laboratory since their arrival in 2001 in a population cage supporting >1000 individuals with overlapping generations.

All flies were reared at moderate density on standard cornmeal molasses agar medium at 25°C and a 12L:12D cycle. Males and females were collected from culture bottles as virgins following light ether anesthesia and stored 10 flies per 8-dram vial with medium inoculated with live yeast until reaching experimental age.

Sperm and SR Dimensions

For some experimental analyses, sperm length and SR length were treated as discrete factors (e.g., long- versus short-sperm line). In other cases, it was necessary to measure mean sperm head or total length for individual males and SR length for individual females. Sperm of each anesthetized male were measured following dissection of the seminal vesicles into phosphate-buffered saline (PBS) on a subbed slide. After passively releasing a few hundred sperm into the saline,

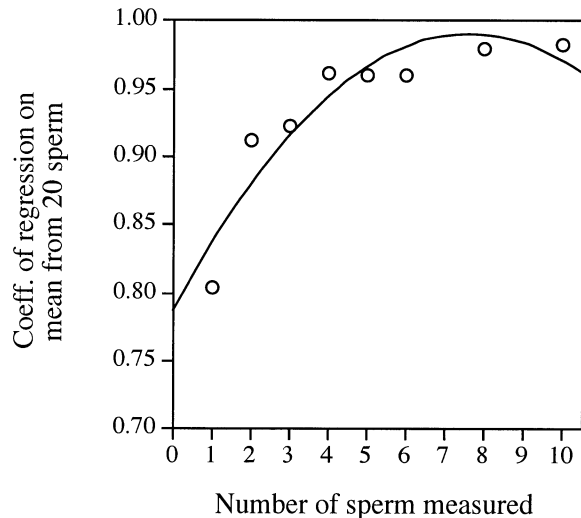


FIG. 1. Relationship between the number of sperm assayed and the accuracy of estimation of male sperm length.

preparations were dried in a 60°C oven, fixed in methanol:acetic acid (3:1), stained in a 5×10^{-7} M solution of Hoechst 33258 (Sakaluk and O'Day 1984) and then mounted with glycerol:PBS (9:1) under a glass coverslip. Digital images of sperm were obtained using a Dage CCD72 camera (Dage-MTI Inc., Michigan City, IN) mounted on an Olympus BX60 microscope (Olympus America Inc., Melville, NY) and lengths were measured using NIH Image public domain software (<http://rsb.info.nih.gov/nih-image>). Total sperm length was quantified using darkfield optics at a magnification of 200× and sperm head length using epifluorescence at 1000×.

Prior to examining the mechanisms conferring a fertilization advantage to relatively long sperm, it was necessary to discern (1) population (selection line) differences in the mean and variance of sperm length, and (2) the relationship between sperm head length and total length. We thus measured both head and total length for each of 20 sperm per male ($n = 15$ males per line). These data confirmed that within-male variation in sperm length was low (inferred from analysis illustrated in Fig. 1), that the long- and short-sperm lines exhibit nonoverlapping distributions in total sperm length (Fig. 2) and that these populations also differ significantly in the length of sperm heads (Fig. 2). Thus, these lines could be experimentally used to explore the contribution of sperm quality and quantity to differential male fertilization success and the mechanisms underlying the demonstrated advantage of relatively long sperm (Miller and Pitnick 2002).

Seminal receptacle length was determined for each anesthetized female by dissecting the reproductive tract into PBS on a microscope slide, paring away extraneous tissue with fine probes, and severing the tracheoles binding together the loops of the SR. A glass coverslip with clay at the corners was then placed on top of the specimen, and the clay was carefully compressed, while viewing through a microscope, until the SR was flattened to two dimensions, but without overcompressing and thus stretching the organ. The preparation was then viewed and a digitized image captured at 200× using differential interference contrast microscopy. Using NIH Image, diameter of the SR lumen was measured

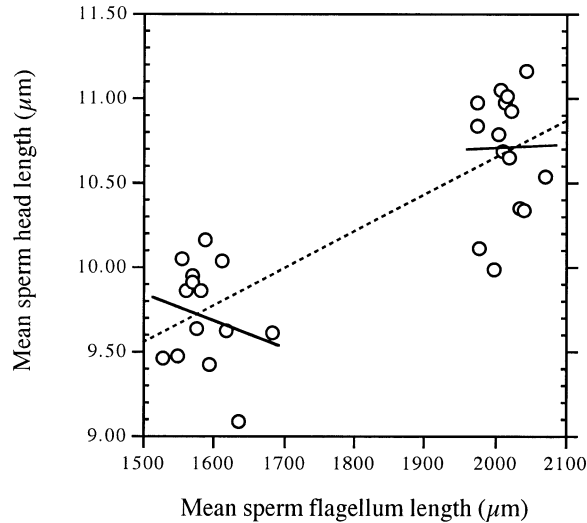


FIG. 2. Relationship between mean (per male) sperm head length (μm) and mean (per male sperm flagellum length (μm) ($n = 20$ sperm per male). Best-fit lines from least squares regression are shown for analyses of discrete selection lines (solid) and for both lines combined (dashed).

approximately every 0.10 mm and SR length determined by tracing the lumen from proximal to distal ends.

Contribution of Sperm Quality and Quantity to Competitive Fertilization Success

The contributions of sperm quality and quantity to male competitive fertilization success were determined by assaying second male sperm precedence (P2, arcsine square root transformed) while factorially varying the quality (short versus long) and quantity (few versus many) of sperm transferred by second males. Sperm quantity was manipulated by varying the number of copulations performed by the male prior to

the experimental copulation (Fig. 3). All females were initially mated to an LH_M - BW male and then remated after three days to a wild-type (long- or short-sperm selection line) male transferring either (1) many long sperm, (2) few long sperm, (3) many short sperm, or (4) few short sperm ($n = 20$ per treatment; Fig. 4), with females randomly assigned to second male treatments. Only females from the long-SR selection line were used for this experiment, because these demonstrate the greatest level of sperm choice in favor of longer sperm (Miller and Pitnick 2002).

The general design of the experiment was identical to that used by Miller and Pitnick (2002). Virgin four- to six-day-old females were initially mated and then remated to an experimental male three days later. Females were transferred to fresh vials containing media and live yeast immediately following remating. They remained in these vials for 24 h and were then transferred to a second vial for 24 h before being discarded. After all progeny had eclosed, paternity was ascertained by eye color and P2 was calculated as the proportion of offspring sired by the second male. The number of progeny eclosing from vials occupied by each female prior to remating was quantified and this variable (an index of the number of first male sperm used by the female prior to remating) was entered as a continuous covariate in the statistical analysis of P2.

Two preliminary experiments were conducted to determine the appropriate number of prior matings to subject long- and short-sperm males to manipulate sperm quantity. The number of sperm transferred by males was assayed directly in one experiment by counting the number of sperm ejaculated into each of five successive control-line females ($n = 5$ males per line; Fig. 3A) and indirectly in a separate experiment by counting the number of progeny produced by six successive control-line mates ($n = 20$ males per line; Fig. 3B). In both experiments, each male was paired with a virgin female and transferred to a vial containing a new virgin female immediately following termination of each successive copulation.

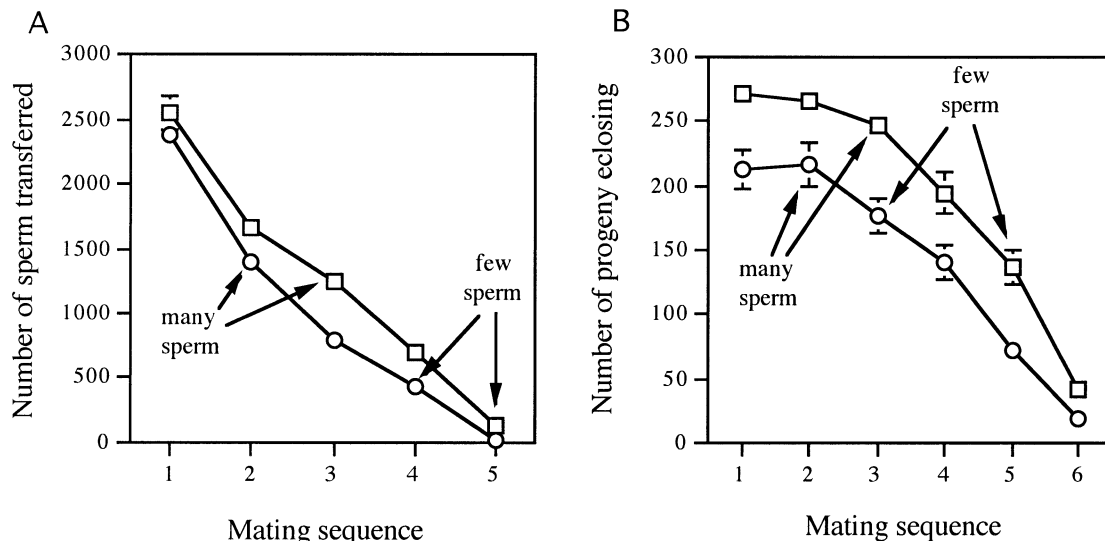


FIG. 3. Number of sperm transferred (A) and number of progeny eclosing (B) across a succession of matings by individual males from the short-sperm (circles) and long-sperm (squares) populations. Bars indicate 1 SE (bars are too small to be visible for some points).

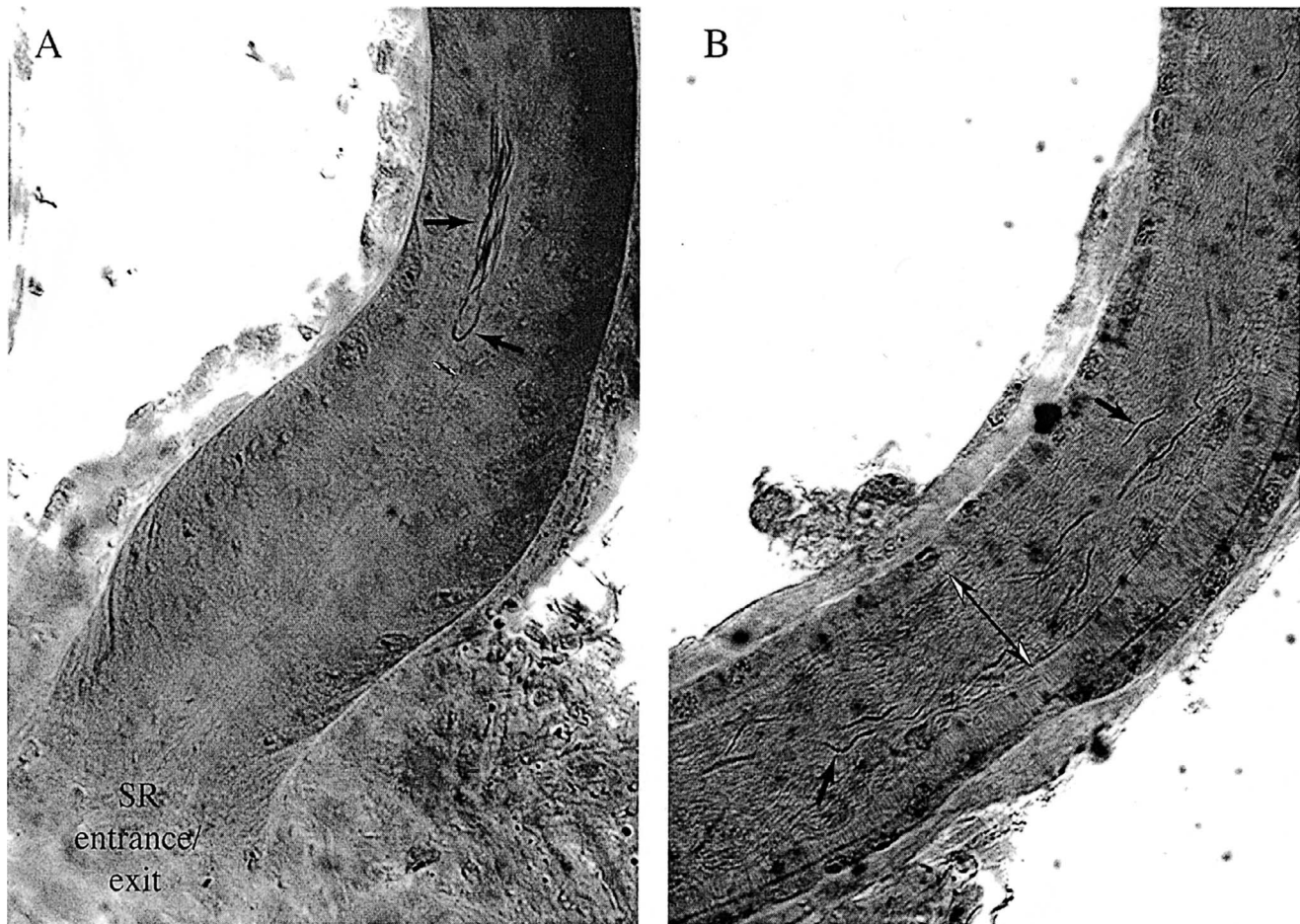


FIG. 4. Micrographs showing in vivo organization of aceto-orcein stained sperm heads within the proximate (left) and distal (right) ends of the female's seminal receptacle. Both images were obtained at the same magnification. Arrows indicate select sperm; double-headed arrows indicate diameter of SR lumen. Because these are optical slices, only sperm heads positioned within the depth of field are visible.

For sperm transfer, females were frozen immediately following male dismount and were later thawed and the sperm were dissected into PBS from the bursa copulatrix (uterus), seminal receptacle, and paired spermathecae (the vast majority of sperm were in the bursa), then dried, fixed, stained, and counted under epifluorescence microscopy at $400\times$. For progeny production, each female was initially retained in the vial in which mating took place, transferred to a fresh vial on days 2, 4, and 6 henceforth, and discarded on day 10. All progeny eclosing from these vials was quantified.

It was important to confirm that long-sperm males, in both many and few sperm treatments, transferred no more sperm than short-sperm males. Otherwise, a statistically significant effect of the sperm length factor could arise but in fact be attributable only to a sperm quantity effect. Additionally, to avoid comparing the fertilization success of "virgin" males with that of previously mated males, all males inseminated at least one female prior to being used in an experimental mating. Long- and short-sperm line males in the many-sperm treatments mated twice or once, respectively, prior to the experimental mating, and in the few-sperm treatments they mated four or five times, respectively,

prior to the experimental mating (Fig. 3A). This protocol was conservative in that any asymmetry in the number of sperm transferred was biased in the direction of long-sperm line males transferring fewer sperm than did short-sperm line males (Fig. 3A).

Organization of Sperm within Females

We quantified how sperm from long-sperm and short-sperm line males became organized within the seminal receptacle of twice-mated females through two separate experiments. First, the general organization of sperm throughout the SR, independent of line of sperm origin, was established by mapping the position of every sperm within the seminal receptacle in vivo ($n = 20$ females evenly distributed across two female treatments [short-SR and long-SR selection lines] by two male order treatments [long-sperm line male first and short-sperm line male second and vice-versa]), using the identical protocols and timing of assay to that used in the sperm precedence experiment described above (and used in Miller and Pitnick 2002). Twenty-four hours following remating, females were flash frozen in liquid nitrogen

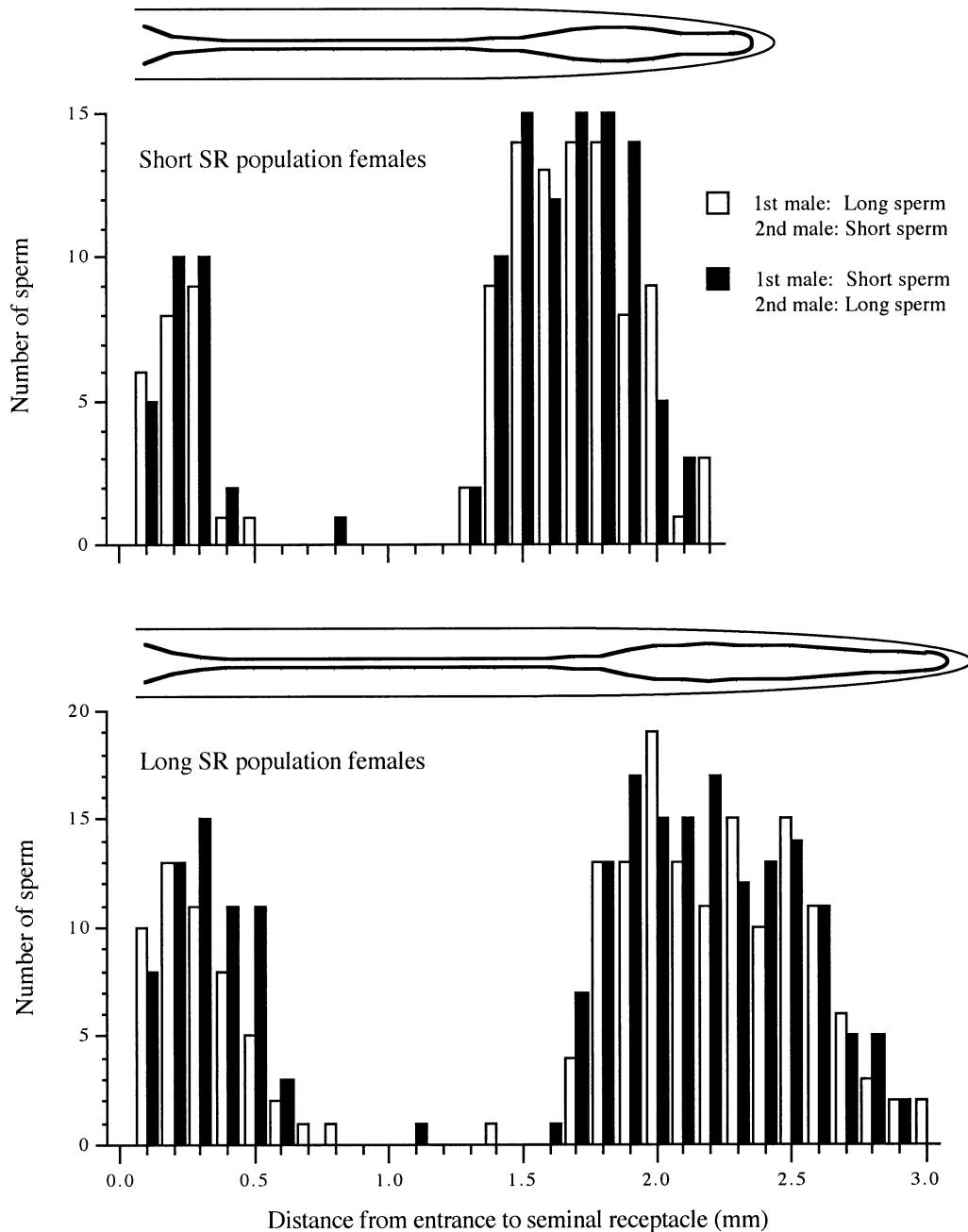


FIG. 5. Frequency histograms mapping the distribution of sperm heads throughout the female seminal receptacle for both (A) short-SR population and (B) long-SR population females. All females were doubly mated, either first to a long-sperm male and next to a short-sperm male (white bars) or vice versa (black bars). See text for details of the mating and dissection procedure. Distance 0.0 indicates the proximate end (i.e., entrance/exit) of the seminal receptacle and the approximate site of egg fertilization. Positioned above each histogram is a schematic illustrating the dimensions of the inner diameter of the lumen for the respective female lines. Each schematic is accurately positioned relative to the x-axis of the respective histograms.

and then frozen to the surface of media at -20°C until dissection. The SR of females was later dissected out, fixed, and stained with 2% orcein in 60% acetic acid (Gilbert 1981; Gilbert et al. 1981). The absolute number of stained sperm heads residing within each consecutive 0.10-mm long section of the SR were counted across the entire organ at $400\times$ using differential interference contrast microscopy (Fig. 4). The diameter of the SR lumen was also measured every 0.10 mm

($n = 10$ females per line) to assess any morphological variation covarying with the pattern of sperm distribution (Figs. 4 and 5). This experiment established that sperm adopt a nonrandom, bimodal spatial frequency distribution across the proximal and distal regions of the SR (Figs. 4 and 5).

In the second experiment, each female was mated to one long-sperm and one short-sperm line male, and distribution of sperm from the two competing males within each female's

SR was quantified by estimating the proportion of both sperm types within the proximal SR and distal SR sperm subpopulations. This experiment used the identical four mating treatments ($n = 30$ females per treatment), protocols, and timing of assay described above for the sperm organization and sperm competition experiments. Again, females were frozen in liquid nitrogen after 24 h and then frozen to the surface of media at -20°C to await dissection. The SR of these females was later dissected into PBS containing 0.10% Triton-x. A dissection technique was employed that results in removal of all sperm from the SR as a single, intact, ropelike mass without altering the relative position of sperm within the mass (Fig. 6). These preparations were dried, fixed, stained and mounted. Under these conditions, it was not possible to measure the total length of individual sperm. However, the length of sperm heads could be accurately measured, and this was done under epifluorescence at $1000\times$ as described above. For each female, the heads of all sperm occupying the proximal end of each SR were measured, as were a random sample of 100 sperm occupying the distal end of the SR. Due to the challenging nature of the dissection technique, not all dissections were successful and hence final sample sizes of treatments vary ($n = 19\text{--}29$; see Table 4).

Because the respective distributions of sperm head lengths for the long- and short-sperm lines overlap (Fig. 2), we used an expectation maximization (EM) algorithm to estimate the proportions of the two sperm categories (long and short) in the observed mixed distributions (Hasselblad 1966; Ott 1979). The algorithm is implemented in the program NOCOM available from <ftp://linkage.rockefeller.edu/software/utilities/>.

To estimate the proportions, we determined the means and variances of the two categories to be used in the algorithm. Estimates of the variances of each category were obtained from earlier observations on sperm lengths in nonmixed distributions (data illustrated in Fig. 2). These were found to be similar and estimated to be $S^2 = 0.25$. The estimates for the two means were obtained from decomposition of the overall data ($n = 12,181$) using a known common standard deviation (0.5), and unknown proportions (p_1, p_2). The two means were estimated to be $\hat{u}_1 = 9.21$ and $\hat{u}_2 = 10.14$. Throughout the analysis we used these conditions ($\hat{u}_1 = 9.21$, $\hat{u}_2 = 10.14$, common $S = 0.5$) to estimate proportions of the two sperm types in the distal/proximal parts of the SR of (1) each female, and (2) females pooled over each treatment category. Note, however, that when neither means nor proportions were provided, such that both had to be estimated by the NOCOM program, the resulting estimated proportions were nearly identical to those presented.

To evaluate the validity of the mixed-distribution model (two component) as compared to a model based on one component ($u_1 = u_2$, $\sigma = 0.5$) we used a likelihood-ratio (LR) test of the hypothesis that the one-component model provides the same fit as the two-component model, that is, $\text{LR} = -2(L_1 - L_2)$ where L_1 and L_2 are the log-likelihoods of the one-component and two-component models, respectively. In this case, the LR statistic is distributed as a χ^2 with two degrees of freedom when the number of observations is large (Thode et al. 1988). We calculated the LR statistic for the pooled data in each of the four treatment categories and found that

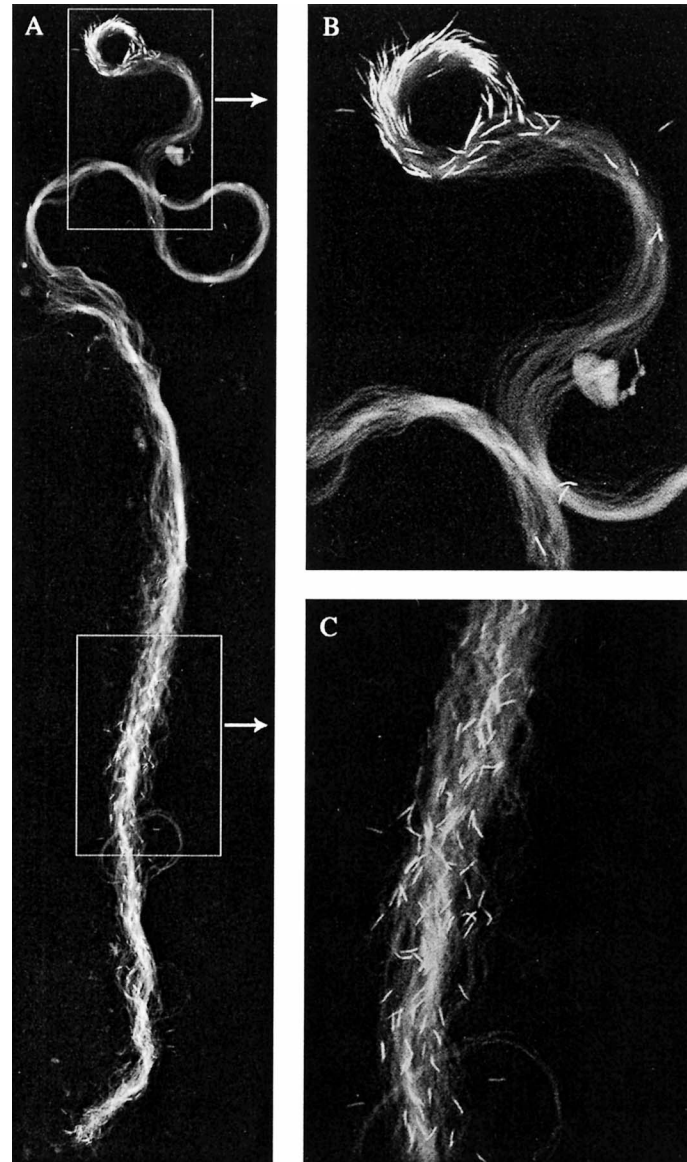


FIG. 6. (A) Micrograph of sperm mass removed intact from a female's seminal receptacle without disrupting the relative position of stored sperm. Fluorescent sperm heads appear bright white. Mass is oriented with the end occupying the proximal end of the SR at the top of the image. (B) Magnified view of proximal end of sperm mass. Note dense clump of sperm heads at proximal end, followed by region containing the tails of those sperm with only a few additional heads. (C) Magnified view of distal region of sperm mass. Note apparent lack of organization of sperm heads.

all showed significant improvement of fit using the two-component model ($P < 0.001$).

We further assessed the efficacy of the decomposition algorithm for estimating the proportions of two sperm populations within sperm mixtures by conducting a simulation experiment. Empirical observations of sperm head lengths for the long- and short-sperm lines (Fig. 2; long sperm $n = 279$, short sperm $n = 265$) were used to create a series of mixed distributions of known proportion of the two sperm types. Each mixed distribution had a sample size of $n = 50$, corresponding to the approximate minimum numbers counted

in samples from the proximate end of the SR of individual females. In the simulation, the proportion (p) of the long sperm type ($1 - p$ for the short sperm type) was specified as 0.10, 0.30, 0.50, 0.70, or 0.90. A random number generator was used to select $n_1 = p \times n$ long sperm and $n_2 = (1 - p) \times n$ short from the empirical datasets; thus, the proportion was known for the mixing process. The proportion of long sperm in the simulated mixed distributions was then estimated using the EM algorithm. Only p was estimated using the original head length means of the long and short sperm (10.67 and 9.73 μm , respectively) and a common variance of 0.25. For each specified p , 20 females (each with 50 sperm) were used to estimate 20 p -values (Table 1).

Note that the bias in the estimate at $P = 0.10$ (in particular) was due to the variance of the short-sperm class in the sample being higher than the value of 0.25 used in the model, whereas the long-sperm class had a variance of 0.25. Because the recommendation for use of the EM algorithm (Hasselblad 1966; Ott 1979) is to use a common variance, we chose to use the smaller of the two empirically determined values, because additional simulations showed this approach to be conservative, with higher variances resulting in greater proportional representation by the longer sperm class.

RESULTS

Variation in Sperm and SR Dimension

There was relatively little within-male variation in total sperm length in the selection lines. An analysis of mean male sperm length based on 20 sperm per male and 15 males per line for the combined long-sperm and short-sperm lines revealed that measuring only a single sperm captures 80.4% of the variation in sperm length within males. Means based on measures of two sperm per male captures 91.2% of the variation, and the number of sperm required to estimate mean sperm length asymptotes at four sperm, with 96.1% of the variation captured (Fig. 1).

A nested analysis of variance on the total length of sperm confirmed a significant difference between the long-sperm and short-sperm selection lines ($F_{1,27} = 1292.98$, $P < 0.0001$); the distributions for the two populations were non-overlapping (Fig. 2). Sperm head length similarly differed significantly between the two lines ($F_{1,27} = 61.52$, $P < 0.0001$), although the distributions largely overlap (Fig. 2).

A correlation analysis of sperm head length and tail length using all data from both the long-sperm and short-sperm lines results in a highly significant relationship between these two characters ($r = 0.588$, $n = 542$, $P < 0.0001$; Fig. 2). However, performing the analysis separately by line reveals no significant relationship between sperm head and tail length within either the long-sperm line ($r = -0.005$, $n = 278$, $P = 0.928$) or the short-sperm line ($r = -0.033$, $n = 264$, $P = 0.592$; Fig. 2). These analyses treated individual sperm as independent, but analyses of mean male sperm head and tail length produced qualitatively identical results.

The total length of the female's SR also differed significantly between the long-SR and short-SR selection lines (Fig. 5), exhibiting nonoverlapping distributions. The SR was found to be a heterogeneous structure, as the diameter of the organ's lumen varied across its length. The lumen of the

organ at its entrance, where it emanates from the anterior-ventral bursa, is relatively wide, with an inner diameter of approximately 27 μm . The lumen in this region appears funnel-like, rapidly narrowing to approximately 7 μm over the proximal 0.3 mm of organ length. The inner diameter of the lumen remains this narrow for approximately 1.1 mm and 1.4 mm in the short-SR line and long-SR line females, respectively. At this point, the inner diameter of the lumen abruptly widens and remains 20–25 μm wide throughout the distal region of the SR, before tapering down to 13 μm at the organ's terminus (Figs. 4 and 5).

Contribution of Sperm Quality and Quantity to Competitive Fertilization Success

Both sperm quality (i.e., length) and sperm quantity contributed significantly to male competitive fertilization success (Table 2). Specifically, both longer sperm and greater numbers of sperm independently contributed to increased male competitive fertilization success. These results thus replicate the sperm quality advantage reported by Miller and Pitnick (2002). There were also three significant interactions: sperm length \times sperm number, sperm length \times prior progeny, and sperm length \times sperm number \times prior progeny. We evaluated the slopes of the interaction terms and determined that none influenced the interpretation of the main effects. The significant sperm length \times sperm number interaction is of particular interest, because it indicates that the advantage in sperm competition afforded by sperm quality increases as the number of sperm competing declines (Table 2; Fig. 7). It is worth noting that line-specific rates of depletion of seminal fluids across successive copulations are unknown.

Organization of Sperm within Females

The distribution of sperm throughout the SR was found to be heterogeneous. There was a spatially bimodal distribution of sperm heads with relatively few heads clustered in the proximate (0.5 mm) end of the organ, followed by a roughly 1.0-mm long section containing virtually no sperm heads, and finally a great many sperm heads distributed throughout the distal end (approximately 40%) of the organ (Fig. 5). The central region lacking sperm heads was not void of sperm, but rather was occupied by the tails of the sperm heads residing in the proximal end of the SR. The transition in the SR, from the lumen containing only the flagella of the proximate cohort of sperm heads to it containing a great many sperm heads in the distal region, is coincident, in both short-SR and long-SR lines, where an abrupt widening of the lumen by approximately four times occurs (described above; Figs. 4 and 5).

Analysis of variance (ANOVA) treating female line (long-SR or short-SR) and male mating order (long-sperm line male first/short-sperm line male second or vice versa) as main effects revealed highly significant effects of female line on the total number of sperm stored in the SR ($F_{1,17} = 83.71$, $P < 0.0001$; mean \pm SE: long-SR line: 200 ± 7 , short-SR line: 117 ± 6), as well as in the number of sperm occupying both the proximate ($F_{1,17} = 105.95$, $P < 0.0001$; mean \pm SE: long-SR line: 52 ± 3 , short-SR line: 26 ± 1) and the distal regions ($F_{1,17} = 42.87$, $P < 0.0001$; mean \pm SE: long-

TABLE 1. Specified proportion (p) of long sperm in mixed distributions, estimated proportion of long sperm by the EM algorithm, and 90% confidence intervals for the estimates for the simulation study.

Specified p	Estimated p	90% CI
0.10	0.16	0.06–0.25
0.30	0.34	0.21–0.42
0.50	0.52	0.37–0.68
0.70	0.69	0.59–0.80
0.90	0.88	0.77–0.97

SR line: 148 ± 8 , short-SR line: 91 ± 6). In all categories, long-SR line females stored more sperm than did short-SR line females (Fig. 5), confirming the report by Miller and Pitnick (2003).

There was no significant effect of male mating order on either the total number of sperm stored ($F_{1,17} = 2.00$, $P = 0.18$) or on the number of sperm in the distal region of the SR ($F_{1,17} = 0.45$, $P = 0.51$). However, there was a significant effect of male order on the number of sperm stored in the proximal region of the SR ($F_{1,17} = 7.38$, $P = 0.015$), with females from both lines storing more sperm in the proximal region when their second mate was a long-sperm line male (Fig. 5). There were no significant female line-by-male mating order interaction effects.

Following the discovery that sperm within the SR are spatially organized into two discrete populations: proximal and distal (Fig. 5), we investigated the contribution of short and long sperm to each of these populations in twice mated females. Using the same four mating treatments described immediately above, sperm were dissected from the SR and the heads of all sperm occupying the proximal end were measured, as were a haphazard sample of sperm heads from the distal region of the SR. These observed mixed distributions of sperm head length data were decomposed using the EM algorithm to estimate the proportions of long and short sperm in three sequential analyses. First, all sperm head length measures from both regions of the SR from all females and all four treatments were combined prior to decomposition to estimate the proportions of long and short sperm that were stored by females. Second, the four mating treatments were analyzed separately, yet within each treatment all sperm head length data from the proximal and distal regions were respectively combined for decomposition analysis. Third, the proportions of long and short sperm found in the proximal and distal regions of the SR were uniquely estimated for each experimental female.

The experiment-wide analysis of all sperm measured generated estimated proportions of 0.32 and 0.68 for the short and long sperm, respectively. Thus, despite each female having been inseminated by one short-sperm line and one long-sperm line male, with both mating orders equally represented, approximately twice as many sperm from long-sperm line males were found to reside within the SR of females. Although these males inseminate more sperm than do short-sperm line males (Fig. 3A, first mating), this difference is not significant ($F_{1,8} = 2.50$, $P = 0.153$, $n = 10$; mean \pm SE: short-sperm line: 2375.4 ± 95.2 ; long-sperm line: $2553.8 \pm$

TABLE 2. Analysis of covariance of second male sperm precedence (P2) for postcopulatory sexual selection experiment with fully factorial variation in sperm quality (i.e., length) and sperm quantity. Prior progeny, number of progeny produced prior to remating by female; df, degrees of freedom; MS, type III mean square.

Source	df	MS	F	P
Sperm length	1	0.178	11.834	0.0010
Sperm number	1	0.136	9.021	0.0037
Prior progeny	1	0.216	14.340	0.0003
Length \times number	1	0.060	4.006	0.0492
Length \times progeny	1	0.077	5.094	0.0271
Number \times progeny	1	0.015	1.013	0.3175
Length \times number \times progeny	1	0.066	4.350	0.0406
Error	71	0.015		

60.6), and could not account for the disparity in number of sperm stored.

In the next analysis, which discriminated among treatments and proximal and distal regions of the SR but combined data for all females with treatments, the proximal region of the SR was estimated to comprise 80–94% long sperm and the distal region 45–78% long sperm (Table 3). Not surprisingly, given the well-established pattern of second-male sperm precedence in *D. melanogaster*, long-sperm-biased proportions were higher when the long-sperm line male was the second mate. It is striking, however, that short-sperm line males do not achieve greater than 55% representation in the distal region of the SR, even when mating second, and they never achieve higher than 20% representation in the proximal region of the SR (Table 3).

In the analyses conducted on a per female basis, estimated proportions of long and short sperm in the proximate and distal regions of the SR reveal an extreme bias in the pattern of sperm storage. Across all four treatments, mean sperm head lengths were consistently longer in the proximal versus the distal region of the SR (Table 4; Fig. 8). Irrespective of mating order, the sperm of long-sperm line males never contribute less than 60% on average to the sperm present in the distal end of the SR, and never less than 83% on average to the sperm in the proximate end of the SR (Table 4). The difference in the representation of both sperm categories between the proximal and distal regions was highly significant ($P < 0.0001$) in all treatments (Table 4). The greatest disparity (22–23% difference on average) between the proximal and distal ends of the SR in the proportional representation of sperm was observed in the two treatments with short-sperm line males mating second. In these two treatments, long sperm

TABLE 3. Treatment-wide number of sperm measured and EM algorithm estimates of the proportion of long sperm in the proximal and distal regions of the SR ($n = 19$ – 29 females per treatment; see Table 4).

Female	First male	Second male	n sperm measured		Proportion long sperm	
			Proximal	Distal	Proximal	Distal
long	long	short	1075	2625	0.82	0.45
long	short	long	1026	2005	0.94	0.72
short	long	short	319	1931	0.80	0.52
short	short	long	719	2481	0.89	0.78

TABLE 4. Mean (actual) sperm head lengths, EM algorithm estimates of the proportion of long sperm in the proximal and distal regions of the SR and of the proportion difference between the two regions, from individual female-level analyses. The F -statistic and P -values are from ANOVAs testing the difference in proportion between proximal and distal regions. Prop., proportion.

Female	1st male	2nd male	n	Mean head length		Prop. (\pm SD) long sperm		Prop. (\pm SD) difference	F	P
				Proximal	Distal	Proximal	Distal			
long	long	short	29	9.97	9.62	0.83 \pm 0.24	0.60 \pm 0.33	0.22 \pm 0.24	57.14	<0.0001
long	short	long	19	10.07	9.88	0.98 \pm 0.03	0.90 \pm 0.09	0.08 \pm 0.09	33.89	<0.0001
short	long	short	21	10.05	9.68	0.90 \pm 0.15	0.67 \pm 0.23	0.23 \pm 0.21	42.84	<0.0001
short	short	long	26	10.15	9.94	0.91 \pm 0.19	0.90 \pm 0.18	0.02 \pm 0.15	18.40	<0.0001

accounted for 60–67% on average of the sperm present in the distal end of the SR, yet accounted for 83–90% on average of the sperm in the proximal end (Table 4). An ANOVA testing the difference between the proximal and distal regions of the SR in the proportion of long sperm ($n = 93$) found no significant effect of female line ($F_{1,89} = 0.30$, $P = 0.5834$), but significant effects of both male mating order ($F_{1,89} = 10.09$, $P = 0.0021$) and the female line-by-male mating order interaction ($F_{1,89} = 4.77$, $P = 0.0315$).

DISCUSSION

Mechanisms of Sperm-Female Interaction

Sperm quality (i.e., length) significantly contributed to male fertilization success (Fig. 7, Table 2). This result confirms the findings of Miller and Pitnick (2002) and supports the conclusion that the relatively long sperm flagella of some *Drosophila* species are the product of sexual selection (Pitnick and Markow 1994; Karr and Pitnick 1996; Pitnick et al. 1999; Miller and Pitnick 2002). Miller and Pitnick (2002, 2003) postulated that sperm quality attributes were likely to coevolve with female reproductive tract design and supported this contention using experimental evolution techniques to reveal significant sperm morphology by female reproductive tract morphology interactions on male competitive fertilization success. Here we identify likely mechanisms underlying this sperm-female interaction, thus revealing the means by which female tract design generates sexual selection on sperm design. Consequently, our results bridge the understanding of microevolutionary process and macroevolutionary patterns for sperm size evolution in *Drosophila* (Pitnick et al. 1999, 2003; Miller and Pitnick 2002, 2003).

The seminal receptacle is the only female sperm-storage organ of many *Drosophila* species and, for those species using both the SR and the spermathecae, the SR is believed to be the primary reservoir of sperm used for fertilization (Pitnick et al. 1999). When an egg descends the common oviduct and enters the bursa to await fertilization, the anterior egg pole with its micropyle (the tube through which the fertilizing sperm must travel) occupies a fertilization chamber at the orifice of the SR (see fig. 1 of Sander 1985). It is therefore reasonable to assume that sperm occupying the proximal end of the SR are better positioned to compete for access to the egg micropyle than are sperm more distally located in the organ, and hence take precedence over them. It is thus relevant that the lumen of the SR was found to be heterogeneous across the length of the organ, being narrow throughout the proximal end and wide in the distal end. This morphology was coincident with a nonrandom distribution of sperm with-

in the organ. Two discrete subpopulations of sperm were found in the SR of all females: a relatively small and well-organized group in the proximal half of the organ and a larger and more haphazardly organized group in the distal half (Figs. 4 and 5).

Two putative mechanisms by which longer sperm achieve a fertilization advantage were identified. First, irrespective of mating order, longer sperm were more likely to be stored in the SR than were shorter sperm. Long-sperm line males contributed over 60% of the sperm in the SR on average when they were first mates, and over 90% on average when they were second mates (Table 4). This effect may be attributed in part to long-sperm line males transferring more sperm per ejaculate than short-sperm line males (see Fig. 3, mating sequence = 1). However, the male line difference in number of sperm transferred was not statistically significant (long-sperm line: 2553.8 ± 60.6 ; short-sperm line: 2375.4 ± 95.2 ; $F = 2.498$, $n = 10$, $P = 0.153$) and so is unlikely to explain the dramatic sperm length effect. Consistent with this biased proportional representation of longer sperm, a greater absolute number of sperm was found in the SR when long-sperm line males mated second (compare black with white bars in Fig. 6). Thus, longer sperm are better at occupying and/or retaining their occupancy in the SR than are shorter sperm. Second, with regard to occupancy in the proximal region of the SR, longer sperm are better at displacing shorter sperm, and better at resisting being displaced by shorter sperm (Tables 3 and 4; Fig. 8). We do not know how having a longer flagellum confers these storage advantages to sperm.

In an earlier report (Miller and Pitnick 2002), the fertilization advantage of longer sperm was observed in long-SR line females only, whereas the distributional effects of sperm length were observed here in both short-SR and long-SR line females (Tables 3 and 4; Fig. 8). We can only speculate on the basis for the different results. With the sperm precedence experiments reported in Miller and Pitnick (2002), short-sperm line and long-sperm line males each competed against control-line males, rather than against one another. Hence, it is possible that even short-SR females are capable of effecting a biased distribution in the current study, given the more extreme disparity in sperm length between competitor males.

It should be noted that mechanisms of sperm precedence examined here in no way preclude the existence of additional factors contributing to differential male fertilization success in *D. melanogaster*. Consistent with our results, numerous reports have suggested that rival sperm displace resident sperm within the female, although this process had not previously been directly observed (Lefevre and Jonsson 1962;

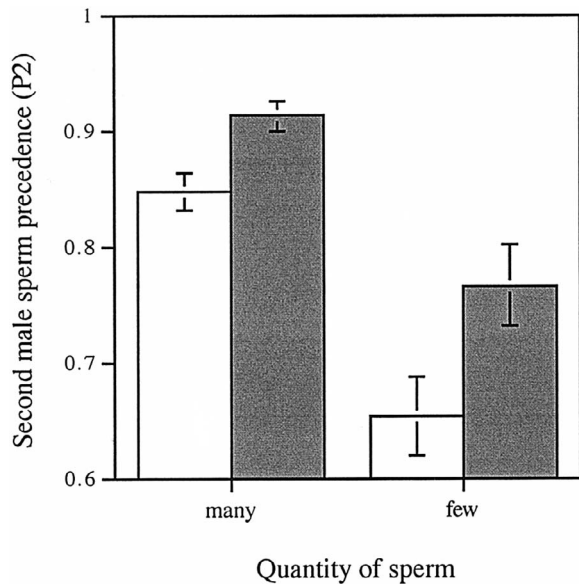


FIG. 7. Pattern of sperm precedence with varying sperm quality and quantity. White columns, short-sperm males; gray columns, long-sperm males. Bars indicate 1 SE. Raw P2 scores shown here are for illustrative purposes only; interpretation is based on ANCOVAs of transformed P2 values (see text for details).

Gromko et al. 1984; Scott and Richmond 1990; Gilchrist and Partridge 1995, 2000; Price et al. 1999). Nonsperm seminal proteins (i.e., Acps) are also known to mediate the fate of sperm within females (Wolfner 1997) and hence are likely candidates to mediate sperm competition (Chapman et al. 2000; Chapman 2001). Acps have been experimentally implicated in sperm incapacitation, but the experimental tests of such an effect have been indirect and the evidence is thus generally unconvincing (Harshman and Prout 1994; Clark et al. 1995; Civetta 1999; Price et al. 1999). Moreover, one claim of having demonstrated sperm incapacitation (Price et al. 1999) was not repeatable by another laboratory (P. Mack, pers. comm.), and direct tests of sperm incapacitation in *Drosophila* (Snook and Hosken 2004) and in humans (Moore et al. 1999) suggest that seminal fluids do not kill rival sperm. Rather, it appears for *Drosophila* that loss of resident sperm is the result of females releasing stored sperm from the SR after copulation with a second male (Snook and Hosken 2004).

Sperm Quality and Quantity Effects on P2

Results of the P2 experiment in which both sperm quality and quantity were independently manipulated indicate that both ejaculate attributes independently influence the pattern of second male sperm precedence in *D. melanogaster* (Table 2, Fig. 7). The preliminary experiment assaying the number of sperm transferred by males from the two lines indicate that the significant sperm quality effect on P2 is unlikely attributable to males from the long-sperm line having transferred greater numbers of sperm. In fact, the test was conservative in that long-sperm line males are estimated to have transferred fewer sperm than did short-sperm line males (Fig. 3A). The magnitude of the sperm quality effect on P2 (Table

2) is striking, especially considering that the sperm quality disparity between treatments was small relative to the sperm quantity disparity. Long-sperm line males produce sperm that are approximately 28% longer than the sperm of short-sperm line males, whereas males from the many-sperm treatments are estimated to have transferred 362% more sperm than did males from the few-sperm treatments.

There was also a significant sperm length-by-sperm number interaction effect on P2 (Table 2) that is attributable to the sperm length effect being greater in magnitude when few sperm were competing than when many sperm were competing (Fig. 7). This result suggests that selection on sperm size will have a positive, self-reinforcing momentum. To the extent that sperm quality trades off with sperm quantity (Pitnick 1996; Oppliger et al. 1998) as a lineage responds directionally to selection for increased sperm quality, the strength of selection will intensify as sperm quantity declines, resulting in species for which males produce relatively few gigantic sperm (Bjork and Pitnick 2006). This interaction may in part explain why, contrary to theory based on Bateman gradients, the opportunity for sexual selection (Wade 1979; Wade and Arnold 1980; Shuster and Wade 2003) does not decline with increasing sperm length (Bjork and Pitnick 2006).

Sperm numbers are predicted by theory to be important to male competitive fertilization success (Parker 1984, 1998), and empirically demonstrated to be important here and elsewhere (Birkhead and Møller 1998; Simmons 2001). Nevertheless, results presented here indicate that, for *D. melanogaster*, sperm quality to a certain extent evolutionarily trumps sperm quantity. Large sperm have significant costs associated with their production (Pitnick 1994), including a reduction in the number of sperm produced (Pitnick 1996), the need for relatively large testes (Pitnick 1996), and delayed male reproductive maturity (Pitnick et al. 1995a,b). For species with giant sperm, the reduction in the number of sperm produced by each male, the increased metabolic cost of growing and maintaining larger testes (Pitnick 1996) and the protracted age at first reproduction in males relative to females, can in extreme environmental circumstances result in sperm limitation within populations (Pitnick 1993; Pitnick and Markow 1994). It has thus remained an outstanding question for such species as to why hypothetical males that mature rapidly and produce many tiny sperm would not have a fitness advantage. The present study suggests that, due to biases imposed by the design of the female reproductive tract, the numerous sperm of such males would be unlikely to enter the population of sperm that have a chance at fertilization.

Most theoretical treatments have modeled sperm competition as either a fair raffle with the probability of a given male siring an offspring dependent only upon the proportional representation of his sperm in the female (Parker 1970b, 1982, 1984, 1990a,b; Parker et al. 1972, 1996, 1997), or a loaded raffle with the sperm from the second of two males competitively weighted as a function of the sperm precedence pattern, but otherwise having fertilization success influenced only by sperm numbers (Parker 1990a; Parker et al. 1997). Two models have made fertilization success dependent both on the size and number of competing sperm. In each, the competitive weight of a sperm increases with its

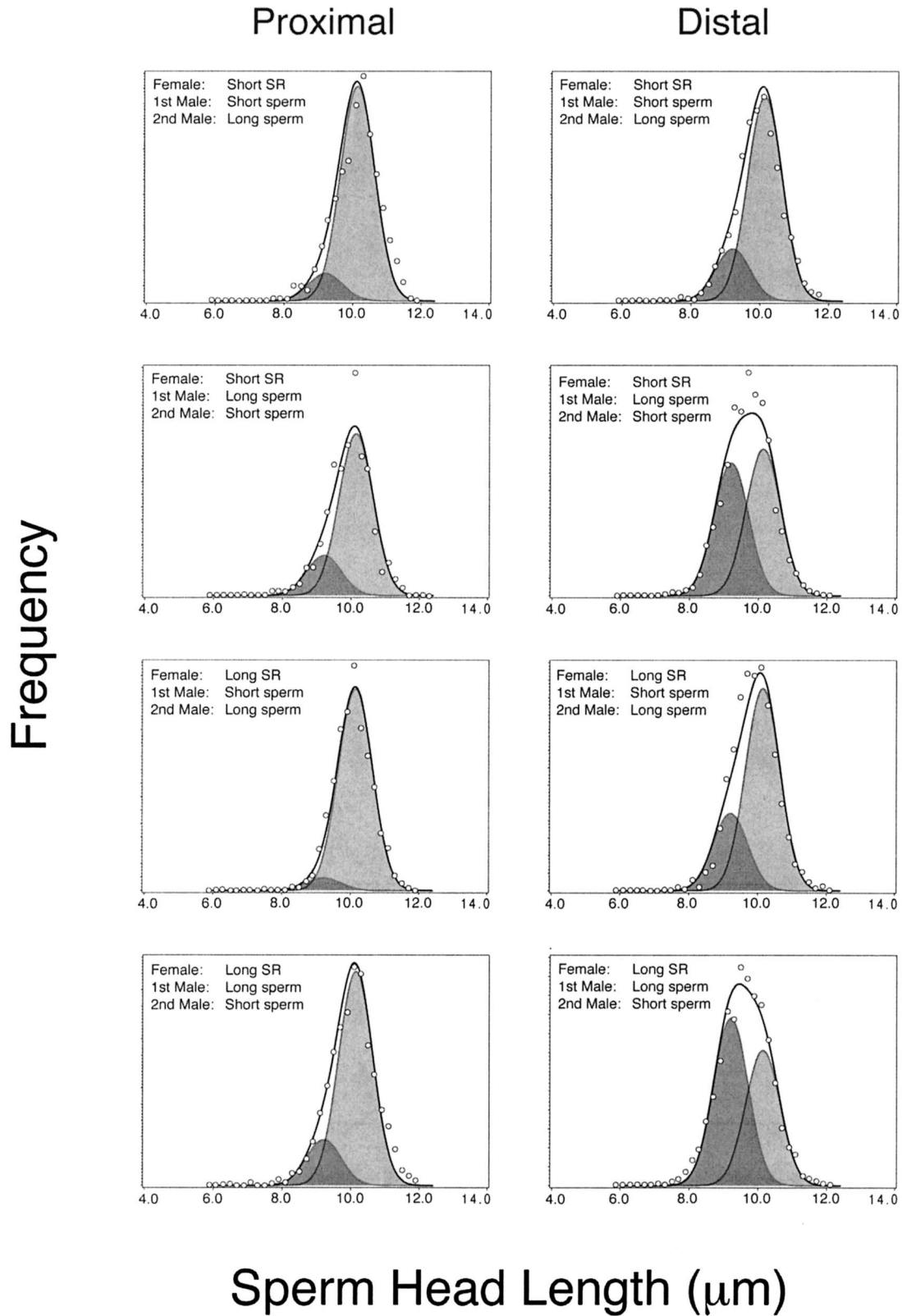


FIG. 8. Decomposition of the distribution for sperm head lengths measured in the proximal (left) and distal (right) regions of the SR for all females from each of the four mating treatments. The empirical distribution of the data is shown by open white circles. Data analyses on each sperm type (measured alone in individual females) showed the distributions of each to be normally distributed with standard deviations of ~ 0.5 . We thus assumed a mixture of two normal distributions each with a variance of 0.25 in the decomposition algorithm. The dark gray distribution represents the sperm from short-sperm line males, the light gray distribution represents the sperm from long-sperm line males, and the white distribution represents the sum of the two distributions.

size, and size and number trade off, either immediately or over evolutionary time. Sperm size is set by the marginal value theorem and is independent of sperm competition risk (Parker 1993; Parker and Begon 1993). With diploid control of sperm size, the analysis (Parker 1993) suggests that increased sperm size will evolve only when the competitive benefits of size become more important as sperm numbers increase or when sperm size correlates positively with sperm longevity. Predicting the evolutionary response in sperm size is more difficult when sperm size is under haploid control (Parker and Begon 1993). A final model examines male ejaculate allocation when females exercise sperm choice (Ball and Parker 2003). However, female choice was defined only as a general discrimination of favorable or unfavorable ejaculates as a reflection of male quality, and thus is not relevant to consideration of sperm form evolution.

Although we have not yet ascertained the relationship between sperm size and longevity, the brief (yet biologically relevant) duration of our experiments and the sperm distributional patterns within females suggest that size-dependent sperm longevity is not the mechanism underlying our results. Coupled with the interactions indicating that sperm quality is more important at low sperm numbers, our results suggest that the assumptions of sperm competition models of sperm size (Parker 1993; Parker and Begon 1993) are not biologically realistic. Further, empirical data demonstrate no contribution of haploid gene expression to sperm length in either *D. melanogaster* or *Scathophaga stercoraria* (S. Pitnick and D. J. Hosken, unpubl. data). The mechanisms of sperm-female interaction identified here, as well as those operating in other species, may prove challenging to address with mathematical models. It is not our intention to suggest that sperm competition models published to date lack utility. Indeed, all else being equal, such as when competing ejaculates contain sperm of equal quality, sperm numbers are expected to substantially influence the competitive outcome. These models thus provide a critical and valuable starting point for elucidating the nature of selection on sperm traits generated by postcopulatory sexual selection. However, it is likely that such models will be more instructive for understanding male total allocation of reproductive effort toward ejaculates (Parker 1993; Parker et al. 1996, 1997; Williams et al. 2005) than for understanding the details of sperm quality and quantity evolution.

Exceptions and Unknowns

Of the numerous comparative analyses that have examined the relationship between sperm size and the risk of sperm competition (see introduction), five studies have failed to find a significant positive relationship. One of these studies was of fish (Stockley et al. 1997) and the remaining four were of mammals (Harcourt 1991; Hosken 1997; Anderson and Dixon 2002; Gage and Freckleton 2003). However, these findings are perhaps consistent with the conclusions of this report, given that most of the fish species included in the analysis have external fertilization, and mammals are unusual in lacking specialized organs and (in most cases) the capacity for prolonged sperm storage by females. With these conditions, the timing of sperm release during a spawn in fish or of

insemination relative to ovulation in mammals (Ginsberg and Huck 1989; Huck et al. 1989) and the number of sperm transferred may be the most important attributes conferring fertilization success upon males. Although longer sperm tails are expected to generate greater propulsive force and hence swim faster (Dresdner and Katz 1981; Cardullo and Balta 1991), the dynamics of motility can differ within the ovarian fluid of a spawn in fish (Turner and Montgomerie 2002) and are expected to be complex and are virtually unknown within female reproductive tracts (Woolley 2003). Moreover, sperm longevity may be an important contributor to fertilization success in fish and mammals. No relationship between sperm length and the longevity of motility was found in a study of Atlantic salmon (Gage et al. 1998). However, this association has not yet received adequate testing (Morrow and Gage 2001a). The more probing question may be why a positive relationship between sperm size and sperm competition was found in another study of fish (but limited to cichlids; Balshine et al. 2001) and in a study of frogs (Byrne et al. 2003), which also predominantly have external fertilization.

It must also be noted that lack of a positive relationship between sperm size and the risk or intensity of sperm competition in comparative studies provides at most only weak evidence against the hypothesis that larger sperm are more competitive. In zebra finches, sperm flagellum length exhibits a negative genetic correlation with the length of the midpiece (Birkhead et al. 2005). As discussed above, sperm size has also been demonstrated to trade off with sperm number (Pitnick 1996; Oppliger et al. 1998) and with life-history characteristics important to fitness (Pitnick et al. 1995a; Pitnick 1996). Given such apparent antagonistic pleiotropy and trade-offs, the balance of selection on complex male phenotypes in a lineage may not favor larger sperm, but this may not mean that, all other things being equal, males producing relatively long sperm would not accrue a competitive fertilization success advantage. Our understanding of net selection on sperm traits is further complicated by issues of possible sex-biased inheritance (Pizzari and Birkhead 2002; Birkhead et al. 2005).

Careful consideration must be given to another exception to the findings presented here and in Miller and Pitnick (2002). Morrow and Gage (2001b) conducted similar experimental evolution studies of sperm length with the cricket, *Gryllus bimaculatus* (which has ~1-mm-long sperm). After five generations of bidirectional selection on sperm length, long-sperm, short-sperm, and medium-sperm (control) line males were competed against one another. In contrast to our results, altering sperm length in this cricket elicited no correlated response in sperm competitiveness (Morrow and Gage 2001a). In a follow-up sperm competition experiment with these selected populations (albeit no further selection beyond the initial five generations), paternity success was assayed relative to continuous variation in sperm length and sperm number among competing pairs of males (Gage and Morrow 2003). In striking contrast to our results, along with a significant positive relationship between sperm number and fertilization success, there was a significant negative relationship between sperm length and fertilization success (partial correlations were conducted to control for any covariance between sperm length and number). There are several dif-

ferences between the *Drosophila* and *Gryllus* projects that may have contributed to the contrasting results. First, although both selection programs produced nonoverlapping sperm length distributions between experimental populations, the extent of this divergence was greater in the *Drosophila* study (28% versus 4.5%). Second, only sperm length was experimentally manipulated in the *Gryllus* study, whereas both sperm length and the interacting component of the female reproductive tract were manipulated in the *Drosophila* study. To the extent that male-by-female interactions determine the relative fertilization success of competing males (Otronen et al. 1997; Clark et al. 1999; Miller and Pitnick 2002, 2003; Amitin and Pitnick 2006), it is unclear what outcome to predict from altering the trait of only one sex. Third, unlike the elongate SR of *D. melanogaster*, female *G. bimaculatus* store sperm in a spheroid spermatheca, which may better promote sperm mixing and sperm use in proportion to their numerical representation within the spermatheca (Walker 1980; Simmons 1987). Nevertheless, interpretational caution is warranted until more work can be conducted on these and other systems.

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